

PAT. Appl.

IN-51

87669

NASA CASE NO.: MSC-21843-1-NP

PRINT FIG: 2

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Serial Number: 07/803,828

Date Filed: December 09, 1991

JSC

(NASA-Case-MSC-21843-1-NP)
THREE-DIMENSIONAL CULTURED GLIOMA CELL LINES
Patent Application (NASA) 28 p CSCI 06C

N92-24052

Unclass

63/51 0087669

THREE-DIMENSIONAL CULTURED GLIOMA CELL LINES

The invention relates to three-dimensional glioma spheroids produced *in vitro*.

Three-dimensional glioma spheroids have been produced *in vitro* with size and histological differentiation previous unattained. The spheroids were grown in liquid media suspension in a JSC Rotating Wall Bioreactor without using support matrices such as microcarrier beads. Spheroid volumes of greater than 3.5 mm³ and diameters of 2.5 mm have been achieved with a viable external layer or rim of proliferating cells, a transitional layer beneath the external layer with histological differentiation and a degenerative central region with a hypoxic necrotic core. Cell debris was evident in the degenerative central region. The necrotic centers of some of the spheroids had hyaline droplets. Granular bodies were detected predominantly in the necrotic center.

The external layer was often more than 15 cell layers thick, densely packed, and measured from 100 to 250 microns thick. The transitional region has pleomorphic cells with pleomorphic nuclei. The transitional cells are less densely packed than the external layer and are circumferentially oriented whereas the external layer has rounded cells with round nuclei, almost radially arranged.

The spheroids exhibited active cellular metabolism. Certain spheroids had extensive pleomorphism with giant cell formation and polymorphism and mitosis in the cellular nuclei. The spheroids expressed detectable glial fibrillary acid protein. Fibronectin and vimentin were expressed.

The artificially generated three-dimensional avascular spheroids of the present invention are *in vitro* produced models of prevascular tumors useful for human tumor ontogeny and evaluation of potential anti-tumor therapies. The histological and metabolic resemblance to *in vivo* tumors is superior to earlier attempts to reproduce spheroid growth *in vitro*.

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MSC-21843-1-NP

Serial Number: 07/803,828

Filed: Dec. 9, 1991

THREE-DIMENSIONAL CULTURED GLIOMA CELL LINES

ORIGIN OF THE INVENTION

The invention described herein was made by employees of the United States Government and may be manufactured and used by or for the Government of the United States of America for governmental purposes without the payment of
5 any royalties thereon or therefor.

BACKGROUND OF THE INVENTION

Cell and tissue culture is essential to neurobiological research concerning transformation of neural cells and tumor cell invasiveness. Laerum, O. D., et al., "Monolayer
10 and Three-Dimensional Culture of Rat and Human Central Nervous System: Normal and Malignant Cells and Their Interactions", 2 *Methods in Neurosciences* 210-236 (1990). For example, cultured malignant glioma cells are frequently selected for general neurobiological studies. However,
15 because cell culture cannot replace the complex conditions existing in the brain, it is important that the functions investigated *in vitro* mimic to the extent possible *in vivo* properties of the same cells.

Malignant tumor growth takes place in a three-dimensional fashion. Solid tumor tissue forms and then spreads
20 as single cells into the surrounding normal tissue matrix. Generally, *in vivo* grown tumors consist of a complex of tumor cells, an extracellular matrix and vessels, and a variable number of inflammatory cells. (Laerum, O. D., et
25 al.)

In the *in vivo* state, abnormal vascularization of malignant tumors is associated with the development of microregions of heterogeneous cells and microenvironments.

Sutherland, R. M., "Cell and Environment Interactions in Tumor Microregions: The Multicell Spheroid Model", 240 *Science* 177-184 (April 8, 1988). The cellular heterogeneity of tumors may explain the behavior of growing tumor
5 cell populations with different metastatic propensities and drug resistances. The cellular heterogeneity of tumors is also reflected by variations in the expression of cellular antigens, which can complicate the use of antibodies conjugated with cytotoxic agents and by variations in
10 specific chromosomal abnormalities. (Sutherland, R. M. (1988)) As an example, gliomas are known to be heterogenous tumors, showing a wide range of phenotypic properties within the same primary tumor or continuous cell line. (Laerum, O. D., et al. (1990))
15 *In vivo* tumor growth is characterized by a phase of exponential cell proliferation followed by a phase of declining growth rate associated with an increase in nonproliferating (quiescent) cells and necrotic cells. (Laerum, O. D., et al.; Carlsson, J., et al., "The Fine
20 Structure of Three-Dimensional Colonies of Human Glioma Cells in Agarose Culture", 85 *Acta Path. Microbiol. Scand., Sect. A* 183-192 (1977); Sutherland, R. M., et al., "Growth and Cellular Characteristics of Multicell Spheroids", 95 *Recent Results in Cancer Research* 24-49 (1984)). *In vivo*
25 tumors frequently begin as avascular spheroids having an external layer of densely packed viable proliferating cells, a transitional layer of less densely packed viable cells and a central necrotic region of hypoxic, quiescent cells. (Sutherland, R.M. (1988); Carlsson, J. et.al.,
30 (1984)) Avascular *in vivo* tumor spheroids having a diameter of approximately 2mm are frequently reported. (Sutherland, R.M., (1988)) The external layer of various *in vivo* tumors have been found to be up to 200 μ m in thickness. (Sutherland, R.M., (1988))

Proliferating tumor cells are usually located within a few cell layers of functional blood vessels. Quiescent and necrotic cells are located at progressively greater radial distances from the vessels. Carlsson, J., et al.,
5 "Liquid-Overlay Culture of Cellular Spheroids", 95 *Recent Results in Cancer Research* 1-23 (1984). The radii measured between the vessels and necrotic areas in a variety of human tumors range from approximately 50 to 250 μ m. (Sutherland, R. M. (1988))

10 Angiogenesis generally occurs in tumors that have reached 1 to 2 mm in diameter. (Sutherland, R. M. (1988)) Vascularization facilitates the delivery of nutrients, substrates and oxygen to individual cells, the removal of cellular waste products, and enhances the distribution of
15 therapeutic agents throughout the tumor growth. During prevascular tumor growth, the effectiveness of therapeutic agents may be limited as a result of poor distribution of the agent to mitotic cells. There seems to be a significant influence of the tissue environment on the efficiency
20 of the vascular supply that develops. Data suggests that the intratumor distributions of important substrates such as glucose or other nutrients, as well as growth factors or hormones may also be deficient and heterogeneous. (Laerum, O. D., et al.; Sutherland, R. M. (1988))

25 *In vivo* tumors vary greatly genetically. (Sutherland, R.M. et al., 1984)) Generally, DNA analysis of *in vivo* tumors of similar cell types will demonstrate quantitatively similar degrees of abnormality. Many, though not all, *in vivo* tumors have been shown to be aneu-
30 ploid in nature.

The three-dimensional *in vitro* spheroid tumor model is intermediate in complexity between standard two-dimensional monolayer cultures *in vitro* and tumors *in vivo*. Spheroids are grown in liquid media allowing three-dimensional growth
35 as compared to two-dimensional monolayer cultures grown on

a surface. *In vitro* cultured spheroids demonstrate overall growth characteristics similar to those of tumors *in vivo*, and reflect the growth rates of the same cell types as tumors *in vivo* more closely than do monolayer cultures. (Sutherland, R. M. (1988); Sutherland, R. M, et al. (1984)) *In vitro* cultured spheroids generally consist of three cell layers that are somewhat similar morphologically and histologically to the cell layers found in *in vivo* tumor spheroids.

10 Generally, most of the proliferating cells found in *in vitro* produced spheroids are located in the outer three to five cell layers. (Sutherland, R. M. (1988)) The quiescent cells are located more centrally and may include a significant proportion of cells that are reproductively
15 viable when removed from these environments. (Sutherland, R. M. (1988)) The distance from the periphery at which necrosis occurs varies from 50 to 300 μm . The spheroid colonies are nearly round in shape. The shape and characteristics of the individual cells in the outer and
20 transitional layer vary. (Carlsson, J. et al., (1984))

Although *in vitro* spheroids are more sensitive to alterations in culture environment than are monolayer or single-cell suspension cultures, spheroids provide a model which at different stages of growth is readily manipulated
25 and controlled experimentally. *In vitro* spheroids facilitate studies of contributions of individual environmental factors, or concomitant changes in these, on cellular phenotypic expression. It is probable that cellular changes which can be demonstrated to occur during
30 *in vitro* spheroid growth also occur *in vivo*. (Sutherland, R. M. (1988))

An important influence of *in vitro* spheroid cell heterogeneity, as to the development of quiescent cells, is the influence of nutritional factors, oxygen tension (pO_2),
35 and central necrosis on cellular phenotypic properties.

For some *in vitro* spheroid types, the thickness of the viable rim of cells surrounding the necrotic center correlates with the theoretical oxygen and glucose penetration distance. However, the availability of other
5 important nutrients, such as glucose, play an important direct or indirect role in controlling the development of central necrosis. (Sutherland, R. M., et al. (1984)) Studies have shown that the maximum spheroid size (volume and cell number) at growth saturation is positively
10 correlated with the oxygen and glucose concentrations of the medium. Spheroid growth characteristics and cellular properties are significantly influenced by spheroid cellular metabolism and the microenvironmental conditions during the culture period. (Sutherland, R. M., et al. (1984))
15 Culture conditions influencing spheroid growth include constituents of the culture medium, frequency of replenishment of the medium, the oxygen and glucose concentrations and the convection properties of the culture medium. (Sutherland, R. M., et al. (1984))
20 Techniques have been developed for producing *in vitro* three-dimensional spheroids to mimic *in vivo* tumor cell growth; however, limiting aspects of such techniques are frequently reported. Generally, as growth of *in vitro* produced spheroids progresses, the number of cells that are
25 proliferating decreases, and the proportion of non-proliferating (quiescent) cells increases. (Sutherland, R. M., et al. (1984); Sutherland, R. M. (1988)) When cells become deprived of oxygen, glucose, and other substrates, and when toxic metabolic waste products accumulate, there
30 are steep gradients in these metabolites, and cell death and necrosis will usually occur in the center of the spheroids. The distance from the periphery of the spheroid at which necrosis occurs may vary from 50 to 300 μm , depending on the cell types, the concentrations of
35 substrates in the growth media, and the overall diameter of

the spheroid. (Sutherland, R. M. (1988); Carlsson, J., et al. (1977)) For most types of human tumor cells grown under optimal nutrient and oxygen conditions *in vitro*, the thickness of the viable rims of cells surrounding the necrotic centers of spheroids ranges from 100 to 220 μm . (Sutherland, R. M. (1988))

A principal method for producing spheroids *in vitro*, though not suitable for all cell types, is a stirred suspension culture in Spinner flasks. Some cell types have been used to produce spheroids of up to 1000 μm or larger using this technique; however, most spheroids produced will have a considerably smaller diameter ($\leq 400 \mu\text{m}$). Additionally, cell shedding from spheroid surfaces is seen with spheroids grown in Spinner flasks. Cell shedding from high shear forces likely attributes to the low volume and frequently small diameter cell colonies produced by Spinner flask culture. (Sutherland, R.M., et al. (1984))

Three-dimensional growth of colonies or aggregates of tumor cells in semisolid medium agar, agarose or methylcellulose has also been described. The three-dimensional, proliferative growth *in vitro* starting from single cells, demonstrated in suspension culture or by tumor cells in a semisolid medium is restricted by insufficient diffusion of oxygen and nutrients and by the accumulation of catabolic products. (Sutherland, R. M., et al. (1984))

Human malignant glioma cells grown in agarose culture were found to be morphologically tumor-like, but did not exhibit the massive degeneration in the centers of the spheroid colonies that is seen with other culture methods. (Carlsson, J., et al. (1977)) Central necrosis was only seen in older colonies that had reached a plateau phase of growth. The spheroids grown in the agarose culture only reached diameters of approximately 600 μm . (Carlsson, J., et al. (1977))

Spheroids of glioma cells grown in liquid-overlay culture have been grown to maximum diameters ranging from about 0.9 to 1.2 mm. (Carlsson, J., et al. (1984)). However, spheroids grown using this method will more
5 frequently have diameters of approximately 250-600 μ m. Liquid overlay cultures have been shown to produce spheroids having less than optimal peripheral nutrient and gas exchange. Necrotic zones appeared when the glioma spheroids had reached diameters of about 300 μ m.
10 Thereafter, the size of the necrotic regions continuously increased. The thickness of the viable cell layers reached a maximum at about the same time that necrosis first appeared.

Other methods for producing *in vitro* three-dimensional
15 spheroids have been reported. Systems utilizing a preserved tissue matrix (spheroid grown in a semisolid agar where no shaking is required) observe an initial size reduction and high rate of cell death. (Laerum, O. D., et al.) Modified organ culture methods involving an overlay
20 suspension containing tumor pieces have also been developed. (Laerum, O. D., et al.) Spheroids produced by this method maintained a close similarity to the histology of the original tumor for the entire culture period, including similarity in morphology of the tumor cells and
25 matrix, as well as preservation of small vessels. A limitation of the modified organ culture method is the large areas of necrosis that are characteristic of glioblastomas *in vivo*. In these spheroids, necrotic areas may occasionally be seen, as well as small areas of
30 degenerated tumor tissue. Cells were also shed from spheroids produced by this method. As a result, cell proliferation did not lead to spheroid growth.

The usefulness of spheroids as research vehicles is dependent upon the three-dimensional colonies closely
35 mimicking *in vivo* tumors morphologically, histologically,

and functionally. The presently used methods for producing spheroids have been shown to produce spheroids having particular limitations. Tumor aggregates produced using Spinner flask cultures result in low volume colonies having
5 diameters of less than 400 μm . Furthermore, shedding of the periphery cells occurs, thus preventing increased diameters of the spheroids produced by this method. Spheroids produced in liquid overlay cultures are also generally small (250-600 μm) in diameter and have reduced peripheral
10 nutrient and gas exchange. Similarly, other presently utilized methods for producing spheroids discussed above fail to imitate *in vivo* tumor cells.

Histological criteria of malignancy in human glioma tumors *in vivo* include extensive pleomorphism with giant
15 cell formation as well as polymorphism and mitosis within cellular nuclei, a hypoxic necrotic core, down-regulation of glial fibrillary acid protein (GFAP) expression, and increased expression of fibronectin. Observation of similar criteria within *in vitro* glioma spheroids may qualify the
20 present invention as an *in vitro* model for both human tumor cell biology and the evaluation of innovative antitumor therapies.

Spheroids provide a unique system in which to simulate the conditions of clinical therapy *in vitro*. The three-
25 dimensional arrangement of cells in *in vitro* spheroids can permit various combinations of physical, chemical, and nutritional stress factors to be tested under conditions that resemble those of poorly vascularized cords or nodules in solid tumors. The penetration of cytotoxic substances may
30 be limited by poorly vascularized tumor tissue. It is essential that the spheroids cultured from tumor cell lines be of similar size and dimension to *in vivo* spheroids in order to accurately determine the potential effectiveness of particular methods of therapies. *In vitro* screening of
35 chemotherapeutic agents may utilize multicellular tumor

spheroids of diverse origins for such purposes. For example, an *in vitro* grown and maintained glioma cell line which closely imitates the characteristics of *in vivo* grown gliomas would enable "customizing" cancer therapies to particular types of gliomas, as well as to specific malignant gliomas from individual patients.

SUMMARY OF THE INVENTION

Three dimensional glioma spheroids have been produced *in vitro* with size and histological differentiation previously unattained. The spheroids were grown in liquid media suspension without using support matrices such as micro-carrier beads. Spheroid volumes of greater than 3.5mm³ and diameters of 2.5mm have been achieved with a viable external layer or rim of proliferating cells, a transitional layer beneath the external layer with histological differentiation and a degenerative central region with a hypoxic necrotic core. Cell debris was evident in the degenerative central region. The necrotic centers of some of the spheroids had hyaline droplets. Granular bodies were detected predominantly in the necrotic center.

The external layer was often more than 15 cell layers thick, densely packed, and measured from about 100 to 250μ thick. The transitional region has pleomorphic cells with pleomorphic nuclei. The transitional cells are less densely packed than the external layer and are circumferentially oriented whereas the external layer has rounded cells with round nuclei, almost radially arranged.

The spheroids exhibited active cellular metabolism. Certain spheroids had extensive pleomorphism with giant cell formation and polymorphism and mitosis in the cellular nuclei. The spheroids expressed detectable glial fibrillary acid protein (GFAP). Fibronectin and vimentin were expressed.

The artificially generated three dimensional avascular spheroids of the present invention are *in vitro* produced models of prevascular tumors useful for human tumor ontogeny and evaluation of potential antitumor therapies.

5 The histological and metabolic resemblance to *in vivo* tumors is superior to earlier attempts to reproduce spheroid growth *in vitro*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph of an artificial three-dimensional spheroid with a diameter of 2.1mm cultured from a malignant human glioma cell line.

10

Figure 2 is a 10 μ cross cryosection (magnification = 160X) of an artificial three-dimensional glioma spheroid of astrocytoma cell line of Example 1.

15 Figure 3 is a 2 μ paraffin cross section (magnification = 80X) of an artificial three-dimensional glioma spheroid of astrocytoma cell line of Example 2.

Figure 4 is an increased magnification (magnification = 160X) view of the artificial three-dimensional glioma spheroid of Example 2.

20

Figure 5, is a 320X magnification of cells of the artificial three-dimensional glioma spheroid of Example 2.

DETAILED DESCRIPTION OF THE INVENTION

The artificial three-dimensional glioma spheroids were generated from malignant human glioma cell lines previously grown in anchorage-dependent monolayer culture conditions. Single cell suspensions were prepared and introduced into a bioreactor with liquid media in a low shear environment. The single cells aggregated, compacted and grew to form spheroids up to 2.5mm in diameter. The cellular growth along the periphery of the cell aggregates produced a viable rim.

25

30

A photograph of one of the artificial three-dimensional spheroids is shown in Fig. 1. The spheroid was generated from the anchorage-dependent astrocytoma cell line CS discussed in Example 2. The spheroid shown has a diameter of approximately 2.1 mm after 13 days in culture. The smooth, round characteristic of the spheroid is evident. The spheroid shown is an avascular tumor model for evaluating tumor therapy as well as studying tumor growth and factors affecting growth. Poorly vascularized tumors have been noted as resistant to conventional therapies.

The spheroids of the invention prepared from different cell lines have active cellular metabolism and develop with significant histological differentiation resembling *in vivo* spheroids. The rim or outer layer of cells are dense and dividing. The spheroids have a degenerative central region with a necrotic core. The spheroids have extensive and progressive pleomorphism. These histological criteria are exemplary of glioblastoma. The spheroids are exemplary of the three dimensional tissue growth for various glioma cell lines and is not intended to limit the invention to a particular embodiment.

Human glioma samples were grown and trypsinized to separate the individual cells. A high density inoculum of at least half a million cells per milliliter was preferred. The spheroids were cultured under low shear conditions in liquid media that allowed for catabolic waste exchange and nutrient supply. Specifically, the clinostatic bioreactor used was a High Aspect Ratio Vessel as described in the published patent application by the National Aeronautics and Space Administration in the Scientific and Technical Aerospace Reports Volume 29/Number 9, May 8, 1991, ACC NOS. N91-17531, U.S. Serial No. 625,345, entitled "A Culture Vessel With Large Perfusion Area To Volume Ratio," invented by David A. Wolf, Clarence F. Sams and Ray P. Schwarz and filed on December 11, 1990, which application is

incorporated by reference in its entirety in this specification. Other bioreactors that provide for suspension of the cells with homogeneous nutrient distribution and gas exchange under low shear conditions
5 can be used to generate the artificial spheroids.

After several hours, cells start to aggregate. After a day or two of culture, aggregates form and afterwards compact. Initially, the aggregate is roughly spherical with a ragged surface. The spheroid then compacts
10 increasing in cell density, and the cells in the outer layer show increased mitotic activity. The spheroids develop in a uniform manner. After at least five weeks of culture in a low shear bioreactor, the peripheral layer remained viable and active.

15

Example 1

Astrocytoma cell line designated HBR09 available from the Huntington Medical Research Institute is an example of a suspension of a normally anchorage-dependent cell line used to generate three-dimensional spheroids of the present
20 invention. The cells were independently passaged and cultured from a confluent monolayer grown prior to suspension in the bioreactor. The monolayer cultures were trypsinized in T-flasks for 10 minutes at 37°C with 0.25% Trypsin in 1mM EDTA and media composed of Dulbecco's
25 Modified Eagle's/Ham's F-12 (Gibco BRL Life Technologies, Inc.) (1:1) + 5% fetal bovine serum (Hyclone Laboratories, Inc.) with 100 units/ml of penicillin and 100 µg/ml of streptomycin. The trypsinized cells were centrifuged for 5 minutes at 1000 rpm (20°C) and resuspended in fresh media
30 at 37°C.

A suspension of 1.25×10^7 cells harvested from log-phase anchorage-dependent cells was introduced into the bioreactor in 50 ml of media. A minimum glucose concentration of 250 mg/dL and a pH of 7.2-7.3 were

maintained during the culture period. A 20 ml daily replacement of fresh conditioned culture medium was prewarmed to 37°C and added to the bioreactor. The vessel was rotated to maintain suspension of the spheroids and minimize collisions with the cell wall. When the cells were cultured for more than 21 days in the low shear bioreactor, the cells aggregate and divide to form a spheroid of relatively uniform size with volumes greater than 3.5 mm³.

10 The spheroids were removed from the bioreactor and prepared for histological examination. For paraffin sectioning the spheroids were fixed with 2% paraformaldehyde, washed three times and dehydrated with graded ethanol series and infused with xylene. After 15 paraffin embedding and sectioning, the spheroid sections were deparaffinized and stained with (Harris) hematoxylin/eosin, toluidine blue or oil red O. For plastic sectioning, samples were fixed and dehydrated using the same method and embedded in water-soluble JB-4 (Poly- 20 sciences, Inc.). Samples were sectioned (2μ) on a MT-2 Porter-Blum ultramicrotome and stained with hematoxylin/eosin or toluidine blue. For cryosectioning, the spheroids were fixed, washed and infused with 0.5 M sucrose for 2 hours, washed twice with cold PBS, embedded in Histo-Prep 25 (Fisher Scientific Co.) and rapidly frozen in liquid nitrogen-cooled isopentane. The samples were sectioned on a Reichart Histostat microtome, air dried and stained with hematoxylin/eosin or toluidine blue and examined.

Viable cells were more numerous and more densely 30 packed in the spheroids' outer rim, which was 100-250μ thick, than in the other two layers. Continued cellular growth along the periphery of the spheroids resulted in a proliferating, external, viable rim of cells.

The spheroids with volumes greater than 3.5 mm³ were 35 characterized morphologically from cryosections as

progressively pleomorphic with the appearance of giant multinucleated cells. The spheroids of Example 1 with volumes greater than 4.2mm^3 had extensive pleomorphism with giant cell formation. The central region of the spheroids
5 were degenerative and characterized by small, round dark staining nuclei. The spheroids had necrotic cores containing cellular debris, numerous hyaline droplets and granular bodies.

Figure 2 is an approximately $10\ \mu$ thick cryosection of
10 a spheroid of Example 1. The spheroid shown is approximately 2.1mm in diameter, with a volume in excess of $4.2\ \text{mm}^3$. The three distinct cell layers are seen: an external layer of viable, proliferating cells indicated by bracket 10; a transitional region indicated at bracket 12;
15 and a degenerative central region indicated at bracket 14. The extensive cellular and nuclei pleomorphism is evident. Giant, multinucleated cells are shown as indicated at numeral 18. Spindle cells are also present as indicated at numeral 16. The viable outer layers of cells 10 are
20 characterized by tandem swirling patterns of elongated cells with lighter staining ovoid nuclei and rough plasma-lemma. The viable cells are more numerous and more densely packed in the external layer 10 ($\approx 100\text{-}250\ \mu\text{m}$ thick) than in the transitional region 12 or the degenerative central
25 region 14.

The artificial three-dimensional spheroids of this example were similar to astrocytic neurosurgical specimens. Both the artificial spheroids and cystic astrocytomas and oligodendrogliomas have many hyaline droplets and granular
30 bodies.

Example 2

Cell suspensions have been prepared from an anchorage-dependent astrocytoma cell line CS from Huntington Medical Research Institute, Pasadena, California. The cells were

grown in monolayers and trypsinized as described above. The cells were introduced into the bioreactor and cultured as described in the **Example 1**. The cells readily formed three-dimensional tumor spheroids without any type of support matrix. The cells of Example 2 divided more rapidly than Example 1, indicating more advanced malignancy of the cell line. After 21 days of culture in the bioreactor, glioma spheroids had volumes greater than 3.5mm^3 .

10 The spheroids were examined for morphological and histological characteristics employing the sectioning and staining techniques previously described in Example 1. Figure 3 is a $2\ \mu$ cross section of the glioma cell line of Example 2. Figure 3 shows the spheroidal shape with a relatively smooth surface as a result of the dense aggregation of cells. In the cross sectioning, part of the external layer is not shown. The external layer of cells on the periphery of the spheroid is indicated at 20. The density of the external layer is evident.

20 Beneath the external layer 20 is a less dense transitional layer 22 which was circumferentially oriented. The transitional layer had pleomorphic characteristics including rounded, spindle and giant cells. The nuclei were pleomorphic. Included in the transitional layer 22 were some mitoses as well as some pyknotic cells with small dark staining nuclei.

30 The central region 24 consisted of a necrotic zone containing cell debris and pyknosis. A decrease in the number and size of intra-cellular lipophilic vacuoles was evident in the cells distal to the necrotic core. The histology was indicative of a high-grade astrocytoma. The three histological layers of Example 2 generally correlates to Example 1.

35 Figure 4 is an increased magnification of a spheroid of Example 2. The paraffin section shows the external

layer indicated at bracket 30 of viable relatively monomorphic proliferating cells up to 15-17 layers deep. The external layer 30 is radially oriented predominantly monomorphic cells.

5 The pleomorphic nature of the transitional layer indicated at bracket 32 is more evident in Figure 4. Spindle cells 36, 37, giant multinucleated cells 38, 39, and round cells 40, 41 are found in the transitional layer. The change in cell orientation from radially in the
10 external layer 30 to circumferentially in the transitional layer 32 is seen in Figure 4. The central region is indicated by brackets 34 in Figure 4. The densely stained nuclei are evident of the hypoxic necrotic core.

Figure 5, which is the spheroid of Example 2 shown
15 under greater magnification, shows a giant, multi-nucleated cell 52 undergoing mitotic division. Spindle cells 50 and 51 can also be seen.

DNA analysis involving cell cycle and ploidy determination of Example 2 (CS) *in vivo* collected and *in*
20 *vitro* cultured glioma cells was performed using flow cytometry methods. Handbook of Flow Cytometry Methods, J. P. Robinson, Ed., Purdue University (1990). DNA synthesis of *in vivo* glioma cells was compared to DNA synthesis of *in vitro* cultured glioma cells obtained from the same biopsy.
25 The data was derived from cells in the G₁ phase of the cell cycle.

Because of great variations genetically between types of tumors grown *in vivo*, DNA Index (DI) values serve as an internal comparison of the quantitatively similar degree of
30 abnormality found between the biopsy tumor cells and the spheroids cultured *in vitro* from the same cells.

The DI values reported in Table I for brain biopsy glioma cells are similar to those reported in Table II for *in vitro* cultured spheroids. The similar DI values of 1.21
35 and 1.35 for brain biopsy versus 1.77 and 1.30 for Example

2 spheroids demonstrate that the degree of hyperdiploidy is quantitatively similar between the *in vivo* tumor cells and the *in vitro* produced spheroids.

TABLE I

DNA IMAGE CYTOPHOTOMETRY OF BRAIN BIOPSY GLIOMA CELLS

SPECIMEN	DNA MASS		
	mass pg	area μm^2	DI
CS-BB-1:	8.7	38.1	1.21
CS-BB-1:	9.7	25.3	1.35

CS-BB-1 = BRAIN BIOPSY = ANEUPLOID

CS-BB-1 = BRAIN BIOPSY = ANEUPLOID

The DI values reported in Table II for glioma cells cultured in monolayers indicate a considerably greater degree of hyperdiploidy than that seen with either the biopsy cells or the spheroid cells. The DI values show that the spheroids cultured from the biopsy cells closely mimic the cell kinetics of the *in vivo* cell line from which it was derived.

TABLE IIDNA IMAGE CYTOPHOTOMETRY OF IN VITRO CULTURED GLIOMA CELLS

SPECIMEN	DNA MASS		
	mass pg	area μm^2	DI
CS-ML-7	17.8	144.6	2.48
CS-ML-14	17.9	166.5	2.49
CS-SP-7	12.7	47.9	1.77
CS-SP-14	9.3	35.0	1.30

CS-ML = MONOLAYERS = TETRAPLOID

CS-SP = SPHEROIDS = ANEUPLOID

Immunohistological testing was performed on spheroids generated by both **Examples 1** and **2**. Tissue sections were deparaffinized with xylene for 10 minutes, permeabilized with methanol at -20°C for 30 minutes and rehydrated by a reverse ethanol gradient. Mouse monoclonal antibodies anti-GFAP [clone G-A-5; Sigma Chemical Co.] and anti-vimentin [clone Vim 3B4; Boehringer-Mannheim] were used at a concentration of 10 µg antibody/ml in a 5% skim milk medium in PBS for 4 hours at room temperature. Rhodamine-conjugated rabbit anti-mouse IgG (H & L) [ICN Biomedicals, Inc.] was used as a secondary antibody at a 1:1000 dilution in 5% skim milk in PBS for 45 minutes at room temperature. The expression of glial fibrillary acidic protein (GFAP) was present. Fibronectin expression was elevated as compared to expression in the monolayer cultures.

The embodiment of Example 1 and Example 2 of the present invention is an artificial three-dimensional spheroid of human glioma cells which closely mimics the morphological and histological characteristics of prevascular *in vivo* glioma tumors. The embodiments described in Example 1 and Example 2 are exemplary of the present invention and the invention is not limited by Example 1 and 2. The invention relates to all glioma tumors and cell lines. There may be modifications made to the described invention that are apparent to those skilled in the art.

ABSTRACT

Artificial three-dimensional glioma spheroids with histological differentiation were generated from malignant human glioma cell lines previously grown in anchorage dependent monolayer culture conditions. The spheroids have
5 an external layer of dense, generally radially arranged, viable, proliferating cells. The central region has a hypoxic necrotic core with a transitional region between the external layer and the necrotic central region. The transitional region has pleomorphic cells containing
10 pleomorphic nuclei, with the cells being circumferentially oriented. The central region also has pleomorphic cells containing pleomorphic nuclei. The spheroids generally have a diameter of at least 1.2 millimeters.

MSC-21843-1-NP

FIGURE 1



FIGURE 2

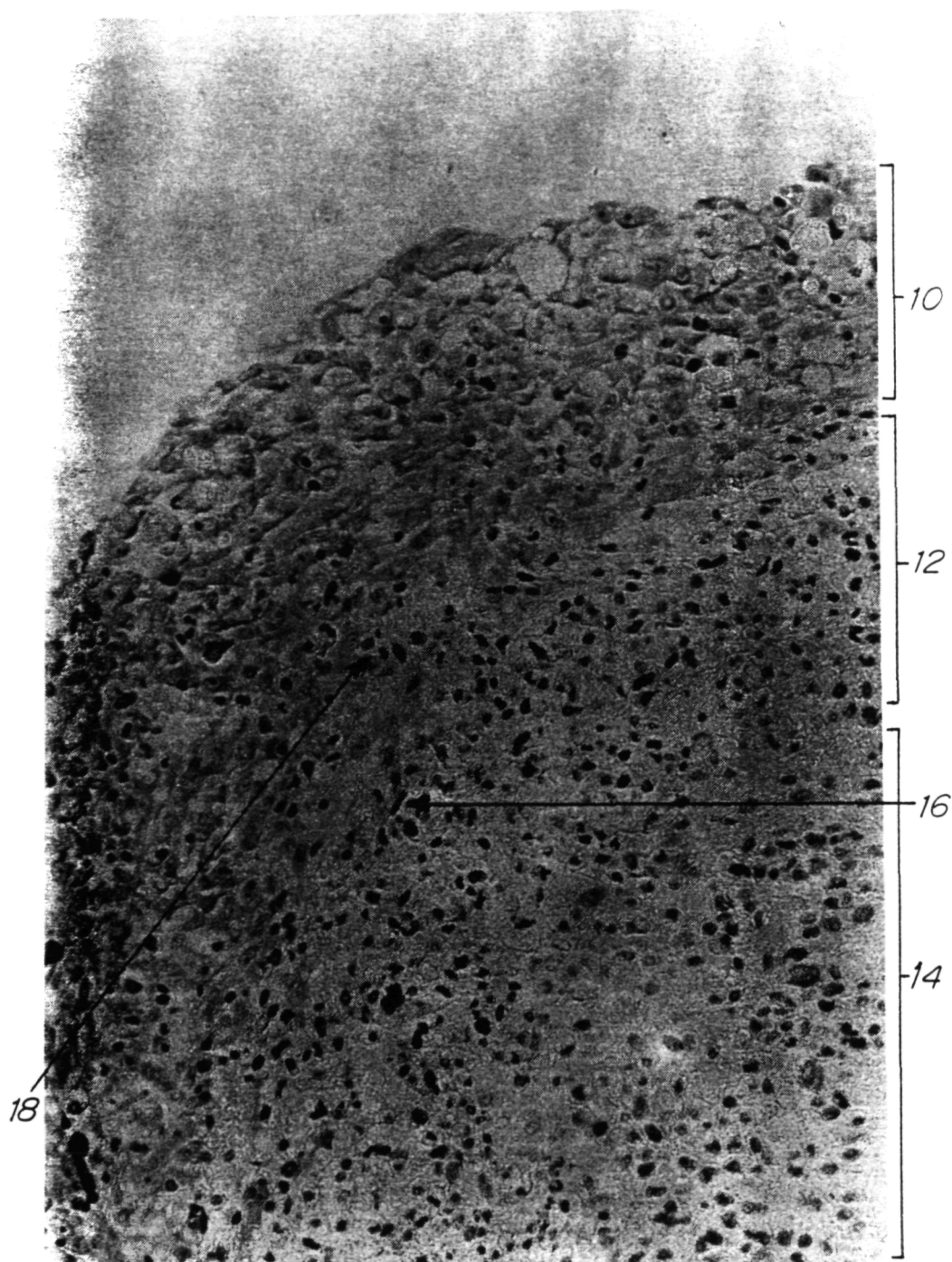


FIGURE 3

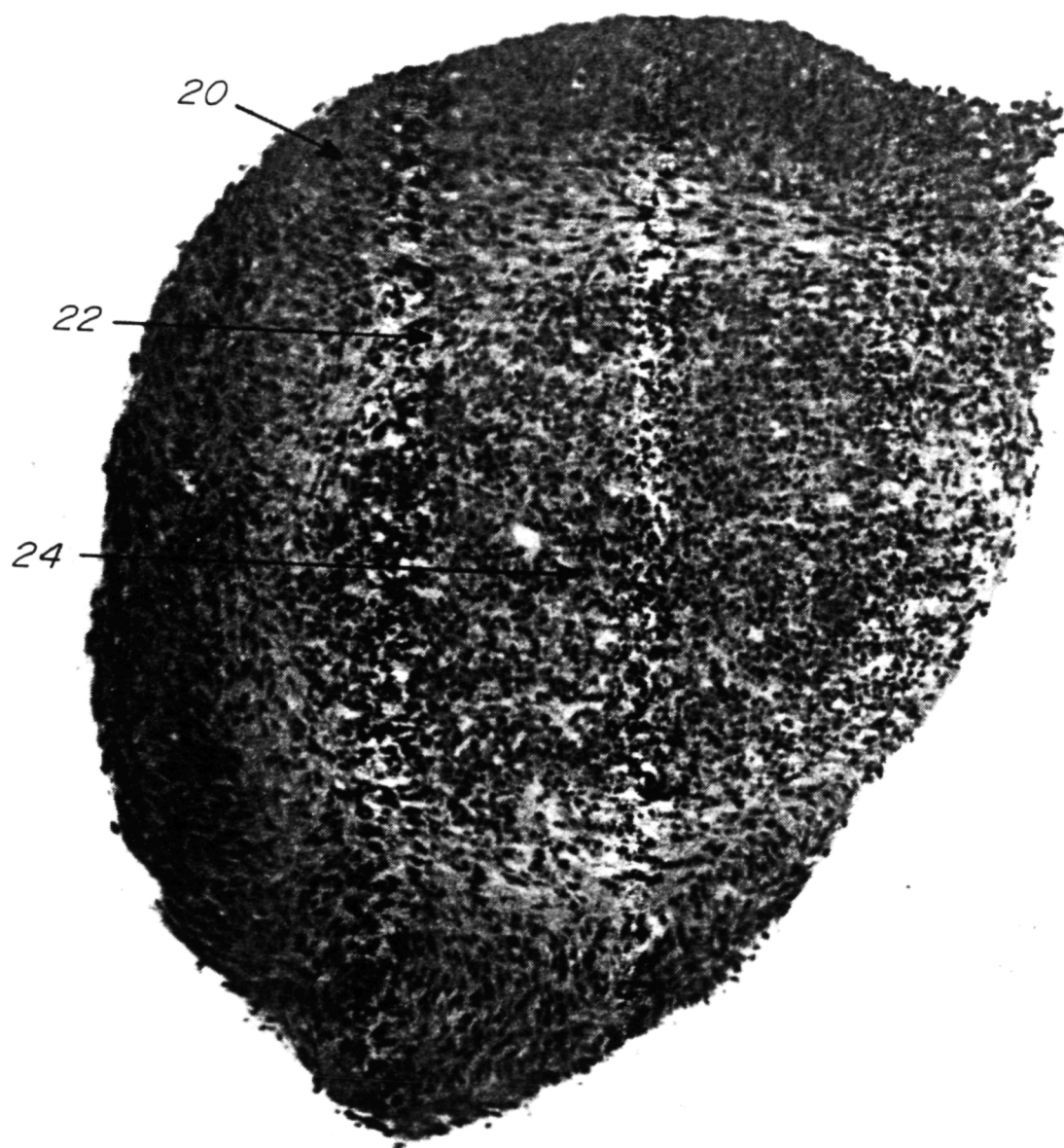


FIGURE 4

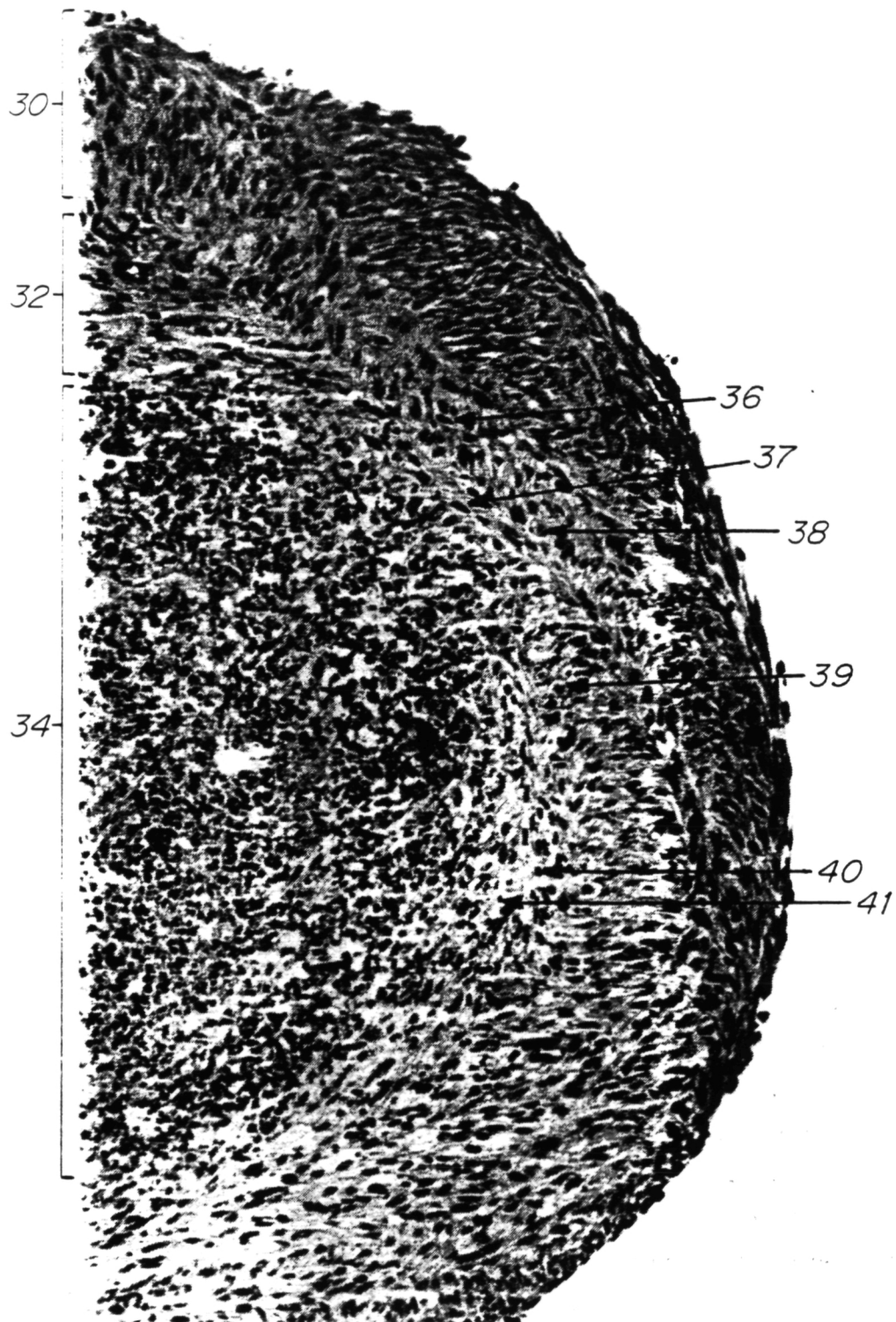


FIGURE 5

